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COMPLETE SPECIFICATION

Method of Treating Hemolytic Streptococci and the Resultant Preparation containing the same

We, CHUGAI SEIYAKU KABUSHIKI KAISHA, a corporation organized under the laws of Japan, of 5—1, Ukima-5-chome, Kita-ku, Tokya, Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a method of increasing the anti-tumor activity and reducing the toxicity of hemolytic streptococci and to preparations obtained thereby.

It is already known that certain living cells of hemolytic streptococci have anti-tumor activity. However, it is extremely dangerous to use living cells in the amount required for treating a tumor, since this bacterium is a pathogen of erysipelas etc. However, it has been reported recently that such pathogenicity (that is virulence or toxicity) decreases and anti-tumor activity increases when cells of hemolytic streptococci having anti-tumor activity at about 37°C are treated for a short time in a medium containing penicillin of a comparative high concentration. ("GANN", Japanese Cancer Association, Vol. 55, p. 233—236, 1964). It has been found that particularly advantageous results are obtained when the cells of hemolytic streptococci are subjected to heat treatment at about 45°C for a short time after initial treatment at about 37°C in a penicillin containing-medium (The Japanese Journal of Experimental Medicine, Vol. 36, p. 161—174, 1966).

Whilst such a treatment using penicillin can increase the anti-tumor activity of hemolytic streptococci and decrease the pathogenicity thereof, the preparation obtained by this method cannot be administered to patients who are susceptible to anaphylactic shock caused by the penicillin. This is an important problem because of recent increases in the numbers of persons sensitive to penicillin as a result of the wide scale use thereof in the treatment of various kinds of diseases. Furthermore, because penicillin and protein are readily combined under alkaline conditions, the protein of pencillin-producing cells causes a rise in the formation of anti-bodies leading to the development of allergies.

Investigations have therefore been carried out to find means of using hemolytic streptococci in the treatment of tumors whereby antibodies are not produced. It has now been found that the addition of cephalosporin C and cycloserine to hemolytic streptococci cells increases the activity of such cells in the treatment of tumors over that of penicillin, and substantially reduces the toxicity of the bacterium, in contrast to streptomycin and many other antibiotics and chemotherapeutic agents which do not increase the activity of hemolytic streptococci at all in the treatment of tumors. Furthermore, long-term administration of cephalosporin C and cycloserine can be carried out without the danger of anaphylactic shock which occurs with prolonged administration of penicillin to persons who are hypersensitive thereto.

According to the present invention there is provided a method of increasing the anti-tumor activity and reducing the toxicity of hemolytic streptococci in which a natural or synthetic cephalosporin C or cycloserine is added to a living cell suspension of hemolytic streptococci having anti-tumor activity, in a suspension medium.

The treatment of hemolytic streptococci according to the present invention may be carried out as follows: cephalosporin C or cycloserine is added to a living cell suspension of hemolytic streptococci which is preferably then heated. The cell suspension is prepared, for example, by cultivating hemolytic streptococci having anti-tumor activity in a medium comprising yeast extract, and, after separation from the medium, suspending the cells separated in, for example, Bernheimer's basal

medium (composition: 675 mg of maltose, 6 ml of a 20% aqueous solution of potassium dihydrogen phosphate adjusted to pH 6.9—7.0 with sodium hydroxide, 12 ml of 2% aqueous solution of magnesium sulphate heptahydrate and 66 ml of distilled water, and abbreviated to BBM below). Cephalosporin C or cycloserine is added to the suspension in a concentration of at least 3×10^{-2} moles per litre. Alternatively cephalosporin C or cycloserine may be initially dissolved in BBM, in which the living cells are subsequently suspended.

The cephalosporin C to be used in the present invention may be either natural or synthetic cephalosporin C. Suitable synthetic cephalosporins C are the anti-bacterially related forms cephalothin and cephaloridine. The suspension medium for the cells may be a salt solution, for example, a physiological phosphate buffered-saline, as well as BBM.

The cell suspension containing cephalosporin C and cycloserine is preferably heated, particularly to a temperature of from 30° to 38°C and, more particularly, to about 37°C. The suspension may be heated for 10—20 minutes, 20 minutes generally being sufficient. The method of the invention is particularly effective if the temperature of the suspension is then raised to 38—50°C, preferably to about 45°C, for 20—60 minutes.

The anti-tumor activity of living cells of hemolytic streptococci is increased in comparison with such cells treated with penicillin and the toxicity of the cells is reduced. When cephalosporin C is used, streptolysin C-forming activity is reduced, such diminished activity often being an undesirable feature of preparations active against tumors.

Hemolytic streptococci preparations thus obtained may be used in the treatment of tumors after proper dilution, and long term continuous administration to patients who are hypersensitive to penicillin is possible.

Examples and experimental examples of the present invention will be shown below.

EXAMPLE 1.

9 g of yeast extract (produced by Ebios Yakuhin Kogyo K.K.) were dissolved in 200 ml of distilled water, adjusted to pH 7.0—7.2 with 10% sodium hydroxide and heated at 100°C for 60 minutes, and the precipitate formed after cooling was filtered. The pH of the filtrate was readjusted with 10% sodium hydroxide, and the solution was further heated at 100°C for 30 minutes and filtered. Distilled water was added to the filtrate to bring it to 300 ml, and the filtrate was poured into sterilized flasks and subjected to steam sterilization at a pressure of 1 kg/cm² for 10 minutes.

A cultivated broth of *Steptococcus hemolyticus* Su strain, ATCC No. 21060 (nearly a virulent strain) which had been cultivated for 20 hours in 15 ml of meat-infusion broth, was added to 300 ml of the above mentioned yeast extract culture medium, statically cultivated at 37°C for 20 hours. The cultured broth was subsequently chilled with ice and centrifuged, and the cells obtained were washed twice with physiological saline and suspended in 15 ml of BBM. The UV— absorbance of this cell suspension at 660 mμ was 7.80.

1 ml of 0.258 M cephaloridine-physiological saline solution was added to 5 ml of the BBM cell suspension and the suspension was heated at 37°C for 20 minutes. The results of anti-tumor tests on the streptococci suspension thus obtained are shown in Experiment 1 and Table 1 which follow:

EXAMPLE 2.

Example 1 was repeated and the suspension to which cephaloridine had been added was further heated at 45°C. for 30 minutes. The streptococci suspension thus obtained showed increased pathological activity over that obtained in Example 1.

EXAMPLE 3.

30 g of yeast extract (produced by Ebios Yakuhin Kogyo K.K.) were dissolved in 500 ml of distilled water, adjusted to pH 7.0—7.2 with 10% sodium hydroxide, heated at 100°C for 60 minutes, and the precipitate formed after cooling was filtered. The pH of the filtrate was readjusted with 10% sodium hydroxide solution, and distilled water was added to bring the total reaction medium volume to 1000 ml. The solution was poured into sterilized flasks and subjected to steam sterilization at a pressure of 1 kg/cm² for 10 minutes. A cultivated broth of *Streptococcus hemolyticus* Su strain ATCC No. 21060 (nearly a virulent strain) previously cultivated for 20 hours in 50 ml of a meat-infusion broth was inoculated in 1000 ml of the above mentioned yeast extract medium, which had been statically cultivated at 37°C for 14 hours. The cultivated broth was subsequently chilled with ice, centrifuged and the cells obtained were washed twice with cold physiological saline, and then suspended in 50 ml BBM.

The UV— absorbance of this cell suspension at 660 mμ was 9.7.

1 ml of 0.258 M cycloserin-physiological saline solution was added to 5 ml of cell suspension in BBM thus obtained, and the suspension was heated at 37°C for 20 minutes to increase the pathological activity of the hemolytic streptococci suspension, which had improved anti-tumor activity.

EXAMPLE 4.

Example 3 was repeated and the suspension to which cycloserine had been added was further heated at 45°C for 30 minutes. The pathological activity of the hemolytic streptococci suspension having anti-tumor activity was increased over that of the suspension obtained in Example 3.

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EXPERIMENTAL EXAMPLE 1.
(anti-tumor test).

Samples of cell suspension obtained by the procedure of Example 1 were diluted 10 fold, 20 fold, 30 fold and 40 fold with BBM.

As comparative tests, suspensions containing penicillin G potassium or dihydrostreptomycin sulphate, a suspension free from antibiotics, and a BBM preparation free from cell suspension were also tested.

Mouse ascites were collected on the 8th day after inoculation of Ehrlich ascites carcinoma cells and cold physiological saline was then added after chilling the mouse ascites with ice and centrifuging for five minutes at 800—1000 r.p.m. The carcinoma cells sedimented were then washed twice with cold physiological saline water and suspended in BBM so that the number of cells were counted to be 6×10^7 per ml. 1 ml of this carcinoma cell suspension was mixed with 3 ml of the above mentioned hemolytic streptococci suspension, incubated at 37°C for 90 minutes, and 0.5 ml of this mixed solution was subsequently injected intraperitoneally to mice. (ddY strain, average body weight about 20 g). 5 mice were used for each group, and survival of mice of each group after the injection was observed, the result of which is shown in Table 1.

TABLE 1

Hemolytic streptococci suspension Survival of Mice

Group	Drug added	Concentration of physiological saline aqueous solution	Dilution numbers	10 days after	20 days after	30 days after	50 days after
I	Cephaloridine	108 mg (potency)/ml (0.258M)	10	5/5	5/5	5/5	5/5
			20	5/5	5/5	5/5	5/5
			30	5/5	4/5	4/5	3/5
			40	5/5	4/5	2/5	2/5
II	Penicillin G potassium	1.6×10^5 units/ml (0.258M)	10	5/5	5/5	5/5	5/5
			20	5/5	4/5	2/5	2/5
			30	5/5	4/5	1/5	1/5
			40	5/5	3/5	1/5	0/5
III	Dihydrostreptomycin sulphate	188 mg (potency)/ml (0.258M)	10	5/5	1/5	0/5	0/5
			20	5/5	2/5	0/5	0/5
			30	5/5	2/5	0/5	0/5
IV	None - Physiological saline only		10	5/5	3/5	1/5	1/5
			20	5/5	2/5	1/5	0/5
			30	5/5	2/5	0/5	0/5
V	Control - (not containing cell suspension, BBM only)			5/5	0/5	0/5	0/5

(Note) Concentration of each drug in physiological saline was adjusted so that it was equivalent to the concentration of the Penicillin G potassium (1.6×10^3 units/ml). The calculation was performed by assuming 1 mg of Penicillin G potassium=1667 units, and 1.6×10^5 units/ml=0.258 M. Moreover the concentration dihydrostreptomycin was calculated on the basis that 3 moles of sulphate were present for 2 moles of the dihydrostreptomycin base.

The results as shown in Table 2 were obtained from mice, 8 for each group, by using, in the same manner, hemolytic streptococci suspensions obtained by the process of Example 2.

TABLE 2

Group	Drug used	Hemolytic streptococci suspension: Concentration of physiological saline solution	Hemolytic streptococci suspension: Dilution numbers	Survival of Mice: 10 days after	20 days after	30 days after	50 days after
I	Cephaloridine	108 mg (potency)/ml (0.258M)	20	8/8	8/8	8/8	8/8
			40	8/8	8/8	8/8	8/8
			60	8/8	8/8	7/8	7/8
			80	8/8	7/8	5/8	5/8
II	Penicillin G Potassium	1.6×10^5 units/ml (0.258M)	20	8/8	8/8	8/8	8/8
			40	8/8	8/8	6/8	6/8
			60	8/8	4/8	2/8	2/8
			80	8/8	3/8	1/8	1/8
III	Control (not containing cell suspension, BBM only)			8/8	0/8	0/8	0/8

EXPERIMENTAL EXAMPLE 2.
(Anti-tumor test).

Preparation of sample: The bacterium was cultivated by the method shown in Example 3 and the cells were suspended in BBM.

1 ml of physiological saline solution of cycloserine, Penicillin G potassium or dihydrostreptomycin sulphate was added to 5 ml of the cell suspension, which was heated at 37°C for 20 minutes, and then further heated at 45°C for 30 minutes and diluted with BBM 2 fold, 4 fold and 8 fold and immediately used in the anti-tumor experiment.

Anti-tumor experiment: ddY strain male mice (average body weight about 20 g) to which Ehrlich ascites carcinoma cells (mouse ascites on 8th day after inoculation) had been intraperitoneally inoculated in a quantity of 10^6 per mouse were used and the above mentioned BBM diluted solution was intraperitoneally injected, 0.8 ml per mouse, once a day for a period of 5 days. 8 mice per group were used and the survival time after administration of the cell suspension to mice of each group was observed. The results of the tests are shown in Table 3.

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TABLE 3

Group	Drug Added	Hemolytic streptococci suspension: Concentration of physiological saline solution	Hemolytic streptococci suspension: Dilution numbers	Survival of Mice: 10 days after	20 days after	30 days after	50 days after
I	Cycloserine	26.4 mg (potency)/ml (0.258M)	×4	8/8	8/8	8/8	8/8
			×8	8/8	7/8	5/8	5/8
II	Penicillin T potassium	1.6×10^5 units/ml (0.258M)	×4	8/8	7/8	6/8	6/8
			×8	8/8	3/8	2/8	2/8
III	Dihydro-Streptomycin sulphate	188 mg (potency)/ml (0.258M)	×4	8/8	2/8	0/8	0/8
			×8	8/8	2/8	0/8	0/8
IV	Control (not containing cell suspension, BBM only)			8/8	0/8	0/8	0/8

Note) Concentration of each drug in physiological saline water was adjusted so that it was equivalent to the concentration of the Penicillin G potassium (1.6×10^5 units/ml). The calculation was performed by assuming Penicillin G potassium 1 mg=1667 units and 1.6×10^5 units/ml=0.258 M. Moreover, the concentration of the dihydrostreptomycin sulphate was calculated on the basis that 3 moles of sulphate were present for 2 moles of the dihydrostreptomycin base.

EXPERIMENTAL EXAMPLE 3.

Experimental example 2 was repeated, the concentration of cycloserine-physiological saline solution being adjusted to 26.4 mg/ml and that of potassium pencillin G-physiological saline solution to the same concentration by weight. The results are shown in Table 4.

TABLE 4

Group	Drug added	Hemolytic streptococci suspension: Concentration of physiological saline solution	Hemolytic streptococci suspension: Dilution numbers	Survival of Mice: 10 days after	20 days after	30 days after	50 days after
I	Cycloserine	26.4 mg (potency)/ml (0.258M)	×4	8/8	8/8	8/8	8/8
			×8	8/8	7/8	5/8	5/8
II	Penicillin G potassium	4.4×10^4 units/ml (=26.4 mg/ml)	×4	8/8	3/8	2/8	1/8
			×8	8/8	2/8	0/8	0/8
III	Control (not containing cell suspension, BBM only)			8/8	0/8	0/8	0/8

EXPERIMENT 4.
(Toxicity test).

Physiological saline solutions of cephaloridine or penicillin G potassium were added in a ratio of 0.2 ml each to 1 ml of a BBM suspension of cells of *Streptococcus hemolyticus* Su-strain ATCC No. 21060 (nearly avirulent strain) obtained in the manner described in Example 1 and heated at 37°C for 20 minutes, and then at 45°C for 30 minutes. 0.1 ml of this treated solution were subcutaneously injected into the rear of each mouse (ddY strain, average body weight about 20 g) and it was observed whether death occurred within 24 hours. The results obtained by using 10 mice for each group are shown in Table 5.

TABLE 5

Treating drug	Number of mice died
Cephaloridine	0/10
Penicillin G Potassium	0/10
without using drug	8/10

EXPERIMENTAL EXAMPLE 5.
(Toxicity test).

0.2 ml of a physiological saline solution of cycloserine or penicillin G potassium having the same concentrations are those used in Experimental example 2 were added to 1 ml samples of BBM cell suspension of *Streptococcus hemolyticus* Su strain ATCC No. 21060 (nearly avirulent strain), obtained in the same manner as in Example 3 and heated at 37°C for 20 minutes and subsequently heated at 34°C for further 30 minutes. 0.1 ml of this solution was intraperitoneally injected in each mouse (ddY strain, average body weight about 20 g) and death within 7 days was observed. The results obtained by using 10 mice for each group are shown in Table 6.

TABLE 6

Treating drug	Number of mice died
Cycloserine	0/10
Penicillin G potassium	0/10
without using drug	8/10

EXPERIMENTAL EXAMPLE 6.
(Streptolysin-S forming ability test).

1 ml of sodium ribonucleate solution (concentration 8%) in BBM was added to 1 ml of a cell treated-solution obtained in the same manner as that of Experimental example 4, incubated at 37°C for 2 hours, and after centrifugation, hemolytic activity of the supernatent was determined using rabbit red corpuscles according to conventional methods. The results of the tests are shown in Table 7.

TABLE 7

Treating drug	Streptolysin-S forming ability
Cephaloridin	<1 (hemolysis unit/ml)
Penicillin G potassium	<1 "
without using drug	2260 "

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WHAT WE CLAIM IS:—

1. A method of increasing the anti-tumor activity and reducing the toxicity of hemolytic streptococci in which a natural or synthetic cephalosporin C or cycloserine is added to a living cell suspension of hemolytic streptococci having anti-tumor activity, in a suspension medium.

2. A method of increasing the anti-tumor activity and reducing the toxicity of hemolytic streptococci in which a natural or synthetic cephalosporin C is added to a living cell suspension of hemolytic streptococci having anti-tumor activity, in a suspension medium.

3. A method of increasing the anti-tumor activity and reducing the toxicity of hemolytic streptococci in which cycloserine is added to a living cell suspension of hemolytic streptococci having anti-tumor activity, in a suspension medium.

4. A method as claimed in any preceding claim wherein the hemolytic streptococci is *Streptococcus hemolyticus*.

5. A method as claimed in Claim 4 wherein the hemolytic streptococci is *Streptococcus hemolyticus* ATCC No. 21060.

6. A method as claimed in any one of Claims 1 to 5 wherein the suspension medium is Bernheimer's basal medium consisting of the following composition:

Maltose	675 mg
20% potassium dihydrogen phosphate aqueous solution adjusted to pH 6.9—7.0 with sodium hydroxide	6 ml
2% magnesium sulphate hepta-hydrate aqueous solution	12 ml
distilled water	66 ml

7. A method as claimed in any one of Claims 1 to 5 wherein the suspension medium is physiological saline containing phosphate buffer.

8. A method as claimed in Claim 2 or any one of Claims 4 to 7 when appendent to Claim 2, wherein natural cephalosporin C is added to the suspension of hemolytic streptococci.

9. A method as claimed in Claim 2 or any one of Claims 4 to 7 when appendent to Claim 2, wherein cephalothin is added to the suspension of hemolytic streptococci.

10. A method as claimed in Claim 2 or any one of Claims 4 to 7 when appendent to Claim 2, wherein cephaloridine is added to the suspension of hemolytic streptococci.

11. A method as claimed in any preceding Claim in which the cycloserine or the natural or synthetic cephalosporin C is added to the suspension in a quantity of at least 3×10^{-2} moles per litre.

12. A method as claimed in any preceding Claim in which the living cell suspension to which the cephalosporin C or the cycloserine has been added is heated at from 30—38°C for from 10—20 minutes.

13. A method as claimed in any preceding Claim in which the living cell suspension to which the cephalosporin C or the cycloserine has been added is first heated at from 30—38°C for from 10—20 minutes, and is further heated at from 38—50°C for from 20—60 minutes.

14. A method for treating hemolytic streptococci substantially as described in Example 1 or Example 2.

15. A method for treating hemolytic streptococci substantially as described in Example 3 or Example 4.

16. A preparation containing non-pathogenic hemolytic streptococci of high anti-tumor activity obtained by addition of a natural or synthetic cephalosporin C and cycloserine to a living cell suspension of hemolytic streptococci having anti-tumor activity, followed by heating at from 30—38°C for from 10—20 minutes.

17. A preparation containing non-pathogenic hemolytic streptococci of high anti-tumor activity obtained by addition of a natural or synthetic cephalosporin C to a living cell suspension of hemolytic streptococci having anti-tumor activity, followed by heating at from 30—38°C for from 10—20 minutes.

18. A preparation containing non-pathogenic hemolytic streptococci of high anti-tumor activity obtained by addition of cycloserine to a living cell suspension of hemolytic streptococci having anti-tumor activity, followed by heating at from 30—38°C for 10—20 minutes.

19. A preparation as claimed in any one of Claims 16 to 18 which has been subjected to a further heat treatment at from 38—50°C for from 20—60 minutes.

20. A preparation containing non-pathogenic hemolytic streptococci, whenever prepared by the process claimed in any one of Claims 2 and 14, and Claims 4 to 13 when appendant to Claim 2.

21. A preparation containing non-pathogenic hemolytic streptococci whenever prepared by the process claimed in any one of Claims 3 and 15, and Claims 4 to 7 and 11 to 13 when appendant to Claim 3.

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